(19)	European Patent Office	
(11)	**EP 1 022 329 B1**	
(12)	**EUROPEAN PATENT APPLICATION**	
(45)	Date of publication and awarding of patent:	04.05.2005, Bulletin 2005/18
(21)	Application number:	00400180.9
(22)	Date of submission:	25.01.2000
(51)	International classification:	C12N 1/14, C12N 9/34 C12N 9/62, C12N 9/24 C12P 7/06, A23K 1/165 // (C12N1/14, C12R1:685)
(54)	Multi-enzyme composition comprising glucoamylolytic, proteolytic and xylanolytic activities and the process to produce it by solid-state fermentation of wheat bran by *Aspergillus*	
(84)	Designated states:	AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE
(30)	Priority:	25.01.1999 FR 9900775
(43)	Date of publication of application:	25.07.2000 Bulletin 2000/30
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<u>Remarks:</u> The patent file contains technical information submitted subsequent to the original patent application and not included in this document.

Description

0001 The invention pertains to a composition comprising glucoamylolytic, proteolytic and xylanolytic activities and the process to produce it by solid-state fermentation of wheat bran using *Aspergillus niger.*

0002 There is a known method for producing ethanol from maize amidon, using an enzymatic procedure comprising a stage of amidon liquefaction using alpha-amylase, aimed at hydrolysing the amidon into dextrine, followed by a stage of saccharification by glucoamylase (also known as amyloglucosidase) aimed at hydrolysing the dextrines into glucose, and finally a stage of fermentation of the latter into ethanol.

0003 The use of the enzymes alpha-amylase and glucoamylase is generally satisfactory if relatively pure amidon milk is used, obtained by wet-grinding the maize, but if the maize amidon is to be replaced with wheat amidon or wheat flour, these two enzymes alone do not lead to satisfactory results due to the presence of hemicellulose, which increases the viscosity of the saccharified flour wort to the point that this creates a problem with the execution of the process. During the saccharification stage, additional enzymes such as cellulase and hemicellulase must be used to reduce the viscosity and to remedy this problem. In addition, it is desirable also to use protease during saccharification, with the aim of hydrolysing the proteins in the flour and thus enriching the wort with soluble nitrogen, in anticipation of the final stage of alcoholic fermentation. The addition of a nitrogen source, which is necessary to increase the effect of the raising agents traditionally used during the fermentation process, may thus be reduced.

0004 All the individual enzymes are commercially available in a purified form, but have the disadvantage of being relatively expensive and thus increase the cost of producing wheat ethanol. Moreover, the compositions must be produced from individual enzymes, which complicates the process.

0005 The article by Abraham et al. "Development of an alternate route for the hydrolysis of cassava flour", Starch Starke, Vol 41, No. 12, 1989, pages 472-476, describes the preparation and utilisation of wheat bran fermented in a solid state with *Aspergillus niger*, as a source of amyloglucosidase for the hydrolysis of manioc flour. The wheat bran is humidified and thermally treated, after which it is fermented in a solid state for 1 to 2 days. The process described by Abraham makes it possible to achieve low levels of glucoamylolytic activity (110-224 IU/g).

0006 Labeille, P. et al: "Comparative study of wheat flour saccharification and ethanol production with two glucoamylase preparations", Industrial crops and products, Vol. 6, No. 3-4, 1 August 1997, pages 291-295, describe the preparation from *Aspergillus niger* stock of a composition called AAI, which has glucoamylolytic, proteolytic and hemicellulase activity and which is used for the saccharification of wheat flour. The article by Labeille, P. et al. does not stipulate the production process for the AAI composition.

0007 There is thus a need for a well-priced composition that combines the glucoamylolytic, proteolytic and hemicellulalytic activities to produce ethanol from wheat flour at reduced cost.

0008 This invention is aimed at meeting this need. The invention concerns a composition in accordance with Claim 1. Preferably, the glucoamylolytic activity must be at least 1500 GU per gramme of dry material and/or

xylanolytic activity of at least 400 XU per gramme of dry material. It is also preferable that the proteolytic activity should be at least 400 PU per gramme of dry material.

0009 The invention also pertains to a process in accordance with Claim 8.

0010 Preferably, the glucoamylolytic activity should be at least 1 500 GU per gramme of dry material and/or xylanolytic activity of at least 400 XU per gramme of dry material. It is also preferable that the proteolytic activity should be at least 400 PU per gramme of dry material.

0011 Preferably, the *Aspergillus niger* stock should be chosen from among the stocks NRRL 3112, ATCC 76061 and stocks obtained from the aforementioned by selection or mutation, if the level of glucoamylolytic activity is to be high. The stock ATCC 76061 is particularly preferred.

0012 If a high level of glucoamylolytic activity is aimed for, the wheat bran used as a raw material should be bran from which the amidon has not been removed. Apart from this restriction, any type of wheat bran may be used. However, it is preferable for the wheat bran to contain a high level (at least 40% by weight) of particles with a size of less than 1 mm.

0013 The characteristics of two suitable brans are given below:

Characteristics	***Bran A***	***Bran B***
Humidity (%)	12,3	19,5
Protein content (% MH*)	13,8	14,8
Amidon content (% MH*)	24,6	21,3
Granulometry		
> 1,25 mm	53,9	0,7
between 1,0 and 1,25 mm	8,1	1,3
between 0,5 ad 1,0 mm	33,3	68,2
between 0,25 and 0,5 mm	3,7	24,6
between 0,16 and 0,25 mm	0,3	2,6
< 0,16 mm	0,7	2,6
%MH = % of humid material		

0014 The wheat bran must be humidified and thermally treated, with a view to pasteurising or sterilising it. It is advantageous that the thermal treatment should not be carried out prior to humidification, as the fermentation results are somewhat poorer if the thermal treatment is carried out before humidification. The thermal treatment may consist of heating, for example in an autoclave. An autoclave treatment of 20 minutes at 120-121°C yields very satisfactory results, but less severe conditions (pasteurisation at 105°C for 15 minutes in a steamer) may also be used. It is equally possible to carry out the thermal treatment of the bran by injecting water vapour, which serves to humidify the bran at the same time.

0015 It is also advantageous to adjust the pH during humidification within the range of 4 to 5,5, with the aim of improving the pasteurising effect of the thermal treatment and the desired fermentation process.

0016 Apart from its sterilisation function, the thermal treatment favours the gelatinisation of the amidon in the wheat bran, and thus the availability of this substrate for the mushroom *Aspergillus niger*, which allows for more efficient fermentation.

0017 The humidification of the bran is important, as the moisture content affects

the fermentation performance. The initial moisture content of the bran is initially adjusted between 50-60%, preferably between 50-55%, of the total mass of the bran and water, and is approximately maintained within this interval during the fermentation process, for example by periodically adding water to compensate for the loss of water into the environment. The phrase "approximately maintained" means that it is tolerable if the humidity rate should vary a little (± 5 %) from the 50-60% interval during a relatively brief period between two successive adjustments of the humidity levels or at the end of the fermentation process. It is advantageous in all cases not to fall below a 45% humidity rate. The humidity rate of the culture medium tends to drop during the culture time, as a result of evaporation caused by an increase in the temperature, which in turn is caused by fungal growth, as the aforementioned medium is a poor conductor of heat. The quality of the water used also plays a not insignificant role. Either good quality tap water or distilled water may be used.

0018 The inoculation of the wheat bran may take place with any appropriate inoculum. An expert in this industry will know the various ways of preparing an appropriate inoculum from the selected stock. The inoculation dose should preferably be at least 1 x 10^7 spores/gramme of initial dry material.

0019 Fermentation may take place in any appropriate reactor. Examples of appropriate reactors are described in the article by A. Durand et Coll., published in Agro-Food-Industry Hi-Tech (May-June 1997, pages 39-42).

0020 Fermentation may take place over a period of 1 to 3 days, preferably for between 30 and 80 hours. Fermentation for less than 1 day will be too incomplete. After 3 days, fermentation has been achieved to the extent that it would not be economically feasible to prolong it. The environmental temperature is typically maintained at between 28 and 38°C, and preferably between 32 and 38°C, which corresponds to the optimal range of activity of the stock of *Aspergillus niger* used in this invention. For this purpose, it is advantageous if the air temperature should be adjusted to between 34-38°C during the first few hours of fermentation to favour the germination of the spores, and then reduced to 28-32°C for the remainder of the fermentation process to contribute to the adjustment of the environmental temperature.

0021 The pH of the fermentation medium is not usually adjusted. If the initial value is close to 6,0-6,4, the pH will drop to 3,8-4,2 during the culture time, and then increase again towards the end. This generally correlates with the sporulation phase of the mushroom. Monitoring the development of the pH gives a good indication of the state of the culture.

0022 The fermentor must be aerated, preferably continuously, to supply the oxygen necessary for fermentation and to avoid an excessive accumulation of carbon dioxide produced during fermentation. Moreover, aeration helps to control the temperature and humidity of the culture medium. The air should preferably be slightly saturated with moisture to prevent the culture medium from drying out. It is difficult to give quantitative indications about the air flow, as it is affected by many variables - particularly the size and geometry of the reactor, the quantity of bran used, etc.. Simple routine tests may be carried out to determine an appropriate air flow in each practical case.

0023 More bran should be periodically added to the fermentor with the aid of a stirrer, such as an arm, blade or spatula, during fermentation, with the aim of avoiding the formation of impermeable masses and to ensure that aeration

takes place as homogeneously as possible throughout the bran mass. However, too vigorous stirring should be avoided, as this may damage the mushroom.

0024 The composition according to the invention is a solid composition, which is particularly useful for the production of ethanol from wheat. It may be directly added to the liquefied amidon (dextrine) obtained during the liquefaction stage, to proceed with saccharification. The glucoamylolytic activity is the most important factor here. Preferably a composition according to the invention with a glucoamylolytic activity of at least 750 GU per gramme of dry material should be used, or even of at least 1500 GU per gramme of dry material.

0025 Another possible utilisation of the composition of the invention concerns the production of monogastric animal feeds, for example for poultry and pigs, based on wheat. In this application, it is the xylanolytic activity that constitutes the most important factor. Thus, preferably, a composition with a xylanolytic activity of at least 400 XU per gramme of dry material should be used in this application.

0026 The composition of the invention may be dried or cooled for storage purposes, if desired.

0027 Drying must take place at a moderate temperature so as not to affect the enzymatic activity. Heating to 40°c in an oven is, for example, deemed to be appropriate. In the case of cooling, however, the humid composition may be kept at low temperatures, for example at -20°C.

0028 In the examples, the various enzymatic activities have been measured, using the following methods:

a) Glucoamylolytic activity

0029 The effect of a glucoamylase (GA) product on an amidon solution is produced by the release of sugars. When heated to 100°C in the presence of 3,5-dinitrosalicylic acid (DNS), these compositions take on a brownish colour, measured at 540 nm using a spectrophotometer (Kontron Instruments, Milan, Italy).

0030 The reaction medium contains:

- Amidon 1% solution: 500 µl
- Citrate 0,1 wadding with pH 4,5: 450 µl
- Enzymatic solution: 50 µl

0031 The reaction takes place for 30 minutes at 60°C (55°C for *Aspergillus orizae* GA). Samples are taken every 5 minutes, mixed with the DNS and placed into a cold bath. They are then heated for 5 minutes at 100°C, rapidly cooled and dosed to 540 nm.

0032 We established these dosaging conditions after studying the effect of temperature and the pH on the activity of our GA preparations. Soluble amidon from Merck (Darmstadt, Germany) was used as a substrate for this enzymatic hydrolysis. The DNS was prepared according to the protocol proposed by P. Bemfeld, Methods in enzymology, 1, 149-158 (1955), which is as follows:

⇒ First dissolve:

- 10 g 3,5-dinitrosalicylic acid
- 200 ml 2-molar soda

- 200 ml distilled water

⇒ Then add:

- 300 g of double sodium potassium tartrate

⇒ Fill up the volume to 1 litre with distilled water once the ingredients have dissolved completely.

0033 Once prepared, this reagent must be stored away from light. The calibration curves were drawn up with glucose as a reference product for the dosage of glucoamylolytic activity or for monitoring the liquefaction/saccharification reactions, as well as with xylose to measure the xylanolytic activity.

0034 The glucoamylolytic activity unit (GU) corresponds to the quantity of the enzyme required to release one micromol of reduced material per minute, using the glucose-dosaging conditions as a reference. The glucoamylolytic activity, calculated with the aid of the formula given below, depends on the initial quantity of dry material (IDM):

$$A = (P/Venz)*(Vferm/Mferm)$$

where:

A is the GA activity expressed in $GU.gIDM^{-1}$ ($\mu mol.min^{-1}.gIDM^{-1}$);

P is the speed at which the glucose equivalents are released in $\mu mol.min^{-1}$;

Venz is the volume of the enzyme solution in ml;

Vferm is the total volume of distilled water used to extract the enzyme solution in ml;

Mferm, expressed in g of IDM, is the initial mass of the dry product from which the enzyme solution is extracted.

b) Proteolytic activity

0035 This dosage has been calibrated using azocasein and the Béinon method, which is described in the work "Protein Purification Methods - a Practical Approach", Harris E.L.V. and Angal, S. (editors), IRL Press, Oxford University Press, 1-66 (1989). The deterioration of this substrate by protease results in the release of nitrogen compounds, which absorb UV up to 340 nm. The development of absorption during the hydrolysis kinetics of this protein indicates the importance of the reaction.

0036 The reaction medium contains:

- A 1% solution of azocasein, pH 5,0: 1000 µl
- An enzyme solution: 200 µl

0037 The azocasein (Sigma, Saint-Louis, United States) is dissolved in 0,1 M acetate with a pH of 5,0. The protease activities have been dosaged at this pH as azocasein is insoluble in acetate at a lower pH level. The enzymatic reaction took place at 60°C. Samples were taken every 5 minutes for 20 minutes and mixed with 5% trichloracetic acid (TCA) to stop the reaction.

0038 One unit of protease activity (PU) corresponds to the quantity of enzyme required to increase 0,01 units by A_{340nm} per minute, as a result of the release of nitrogen compounds under the aforementioned conditions. This activity, calculated according to the formula given below, refers to the initial dry material ($PU.G^{-1}$ IDM) or to the glucoamylolytic activity ($PU.GU^{-1}$):

$$A = (P/Venz)*(Vferm/Mferm)$$

where:

A is the protease activity expressed in $PU.gIDM^{-1}$;

P is the speed at which the nitrogen compounds are released to increase 0,01

units of $A_{340nm}.min^{-1}$;
Venz is the volume of the enzyme solution in ml;
Vferm is the total volume of distilled water used to extract the enzyme solution in ml;
Mferm, expressed in g of IDM, is the initial mass of the dry product from which the enzyme solution is extracted.

c) **Xylanolytic activity**

0039 To demonstrate this enzymatic activity, the GA products were allowed to act on a soluble xylane solution, and the sugars released were measured, using the DNS method.

0040 The reaction medium contains:
- A 1% solution of xylane, pH 4,5: 900 µl
- An enzyme solution: 100 µl

0041 The larch xylane solution (Sigma at 1%) was prepared in citrate with a pH of 4,5 and the reaction took place at 60°C. Samples were taken every 5 minutes for 20 minutes, mixed with DNS and placed into a cold bath. They were then dosaged according to the same protocol as that used for measuring the GA activities, using xylose as a reference.

0042 One unit of xylanase activity (XU) corresponds to the quantity of enzyme required to release one micromol of sugar per minute. This activity refers to the initial dry matter ($XU.g^{-1}$ IDM) or to the glucoamylolytic activity ($XU.GU^{-1}$). To calculate this activity, we have used the formula defined for the calculation of the GA activities, in which:
A is the xylanase activity, expressed as $XU.gIDM^{-1}$ ($\mu mol.min.^{-1}.gIDM^{-1}$);
P is the speed at which xylose equivalents are released in $\mu mol.min^{-1}$.
The other terms of the formula were not modified.

0043 The following non-comprehensive examples are given to illustrate the invention:

EXAMPLE 1 - Selection of *Aspergillus* stock

0044 The capacity of seven different commercially available Aspergillus stocks to produce glucoamylase by fermentation of wheat bran in a solid-state medium was studied in a comparative fashion.

0045 The tests were carried out on 50 g of fermentation medium in an Erlenmeyer flask. The medium consisted of 21,5 g wheat bran, 27,5 g water and 1 g wheat amidon. The initial pH of the medium was between 6,0 and 6,5. The medium was sterilised in an autoclave at 120°C for 20 minutes.

0046 Each medium was inoculated with 2.10^7 spores of the stock to be tested per gramme of initial dry material. The age of the spores was 3 days. Fermentation was allowed to take place for 40 or 50 hours, with the Erlenmeyer flasks placed into an oven with a temperature of 35°C. At the end of the fermentation process, the fermented medium was mixed with 150 ml of distilled water to dissolve the enzymes produced, after which the solution was filtered to recover the enzymatic solution. The solution was centrifuged to eliminate the residual spores and particles, after which the solution was decanted into 100 ml flasks, which were stored at -20°C until the glucoamylolytic activity could be analysed.

0047 The stocks tested and the results obtained are summarised in Table 1 below:

Ref. stock	FMS period (h)	Ac. GA (UG.g-1 MSI)
A. niger ATCC 76060	50	627
A. niger ATCC 76061	50	943
A. niger MUCL 28815	40	710
A. niger MUCL 28816	40	631
A. niger NRRL 3112	50	1056
A. oryzae ATCC 22788	50	903
A. oryzae ATCC 42149	50	881

It may be seen that stock A. niger NRRL 3112, A. niger ATCC 76061 and A. oryzae ATCC 226788 show the best activity in terms of the production of glucoamylase.

0048 Another important characteristic to be taken into account, however, is the stability of the glucoamylase produced. Thermostability tests were thus carried out by subjecting the enzyme solutions to thermal treatments at 55 and 60°C for 30 minutes and by measuring the activity of the glucoamylase at the end of this time. These treatments were done under conditions similar to the conditions used for saccharifying amidon. It was found that the stocks *A. niger* ATCC 76061 and *A. niger* NRRL 3112 resulted in the most stable glucoamylase (100% residual activity after 30 minutes at 55°C and about 50% residual activity after 30 minutes at 60°C), while the stocks *A. oryae* ATCC 22788 and ATCC 42149 resulted in glucoamylase with a 0% level of residual activity after 30 minutes at 60°C and 46% residual activity after 30 minutes at 55°C. This led us to select the stocks ATCC 76061 and NRRL 3112 of *A. niger*. The stock *A. niger* NRRL 3112 turned out to be genetically very unstable (loss of activity after several reproductive cycles), thus the most preferable stock to use is *A.niger* ATTC 76061. It is thus this stock that was used in the following examples:

<u>EXAMPLE 2 - Production of glucoamylase in pilot vats provided by INRA (50 l non-sterile): the importance of pretreating the wheat bran.</u>

0049 Tests were carried out with a 50-litre non-sterile fermentor similar to that described in the aforementioned article by A. Durand et al. (Figure 1) on BCE wheat bran (provided by the distillery Brte Champagne Ethanol, Provins, France). Two preparation methods for the bran were used to obtain 5 kg of culture medium with a humidity level of 55%:

- dry bran: the bran was autoclaved for 1 hour at 105°C and then mixed with water (test F4C3);
- moistened bran: the bran was humidified at 45% in a Petri dish and autoclaved for 20 minutes at 121°C (test F4C4).

0050 In both cases, inoculation took place with 2.10^7 spores.g^{-1} DM and the water content of the media was adjusted to about 55%. They were then fermented on a 10cm thick bed in aerated ovens. During the culture time, the medium was intermittently striated with a spatula to reduce its temperature. During fermentation, the atmosphere was continuously replaced with conditioned air, with the temperature, humidity and flow being that indicated in the tables.

0051 The results are presented in Tables 2 (F4C3 test) and 3 (F4C4 test).

0052 These data allow several conclusions to be drawn:

- The humidification of wheat bran before thermal treatment is necessary for efficient glucoamylase production. After decontamination, the thermal treatment of the wheat bran helps the gelatinisation of the amidon;
- The pH appears to be a good qualitative indicator for the development and production of fungal GA, without making it possible to estimate the quantity of GA to be obtained;
- Slightly stirring the medium (striation) does not alter the production of enzymes;
- The development of the water content during the two fermentation processes indicates considerable drying out of the culture medium, which may be unfavourable for fungal growth.

EXAMPLE 3 - Production of glucoamylase in a pilot fermentor provided by the company FUJIWARA: the importance of maintaining the humidity of the medium during the fermentation process.

0053 This test was carried out with a pilot fermentor sold by the company FUJIWARA, Okayama, Japan and using BCE wheat bran. It differs from the fermentor used in Example 2 in that the diameter of the vat is 0,66 m, as against 0,35 m for the INRA vats. In this fermentor, 20 kg of medium with a water content of 55% were prepared according to the humid bran method described in Example 2 are required to make a culture with a thickness of 12 cm. Stirring is ensured by three constantly rotating vertical arms without blades, which mix the medium in the rotating vat (5-10 minutes/rotation). During fermentation - as is the case with the INRA vats in Example 2 - the gaseous atmosphere is constantly replaced with conditioned air, with the temperature, humidity and flow the same as indicated in Table 4.

0054 During this test (FII), the opportunities for adjusting the humidity were studied. The humidity of the culture was measured at several points, using an infrared apparatus, and the mass of the medium was used to determine the quantity of water to be added to maintain the water content of the medium above 50%.

0055 The results of this FII test are given in Table 4. The results obtained require the following comments:

- FII clearly indicates that the maintenance of the water content between 50 and 55% favours the production of enzymes with 1800 GU/g of DM being released after 44 hours of fermentation, i.e. more than twice the activity obtained during the course of the F4C2 test in Example 2, for which no adjustment was carried out;
- the production of enzymes was stabilised after 44 hours of culture time, which correlates with the appearance of fungal spores, which demonstrates that it is not necessary to continue the culture after this phase;
- satisfactory adjustment of the temperature of the medium to about 35°C may be obtained by a good combination of air-conditioning and stirring of the medium;
- the culture is not in any way disadvantaged by intermittent stirring, using the FUJIWARA fermentor stirring system.

EXAMPLE 4 - Production of glucoamylase in a 50-litre INRA pilot fermentor with stirrer: the usefulness of thermal pretreatment of the bran with vapour and a culture under "sterile conditions"

0056 This pilot fermentor resembles that shown in WO-A-94 18306 and in Figure 4 of the aforementioned article by A. Durand et al. This system makes it possible to treat the bran with water vapour directly in the fermentor, which is the preferred preparation method for work on an industrial scale. The culture is also prepared, inoculated and carried out under sterile conditions, with the exception of the sampling process, thus ensuring that the test is semi-sterile, as opposed to the two preceding examples.

A) Experimental conditions

0057 Nine kg of BCE bran were placed into the fermentor, pre-humidified with 1,5 l of water, then sterilised for 20 minutes at 121°C, with periodic stirring every 5 minutes for 5 seconds. This treatment makes it possible to attain a humidity level of 48%, which was then adjusted to 55% during inoculation.

0058 The bran was inoculated with a koji-type product.

0059 Some 180 g of BCE bran (55% initial humidity), fermented for 4 days at 35°C, were mixed with 3 litres of sterilised water to obtain a suspension of spores, which constitutes the inoculum.

0060 The initial fermentation conditions were as follows:
18,3 kg of culture at a 55% level of humidity and with an initial pH of 5,7;
a bed height of 40 cm;
an air flow of 314 $l.min^{-1}$;
an intake air temperature of 35°C;
relative humidity of the intake air: 95%.

B) Monitoring fermentation

0061 Apart from measuring the pH and temperature of the medium, the percentage of dry material and the production of GA, the development of the culture mass was continuously recorded by the 50-litre fermentor, while for the non-sterile reactor, the culture was weighed at 21 hours and at 42 hours of fermentation.

0062 These mass measurements have a dual purpose:

Maintaining the humidity of the culture by estimating the MS percentage

0063 During fermentation, two phenomena contribute to the reduction of the culture mass, i.e.:
- the medium dries out and this has to be compensated for by adding water;
- the loss of dry material, on the other hand, which is associated with fungal growth.

0064 This loss of dry material is not insignificant, with 20% of the DM being lost within 40 hours of culture time, i.e. 0,5% of the DM per hour if one assumes a linear loss.

0065 On this basis, and knowing the mass of the culture ($M_{(t)}$) at all times, it is possible to deduce the theoretical percentage of DM at Time 1 by means of

the following ratio:

$$\% DM_{theoretical(t)} = \frac{IDM\ (IDM.0,5\%).t}{M_{(t)}}$$

where IDM is the initial quantity of dry material.

0066 When the % DM thus calculated exceeds 50%, sterilised water is added to reduce this percentage to 45%.

Expressing the results per gramme of initial MS

0067 The development of the mass and that of the percentage DM measured, makes it possible to calculate the real loss of dry material (P_{DM}, expressed as a percentage) during the culture time. Thus the quantity of GA thus far expressed in $GU.g^{-1}$ DM may now be expressed in $GU.g^{-1}DM$, using the following ratio:

$$(GU.g^{-1}\ IDM) = (GU.g^{-1}\ DM).(100 - P_{DM})/100$$

C) Results

0068 Tables 5 and 6 summarise the operating conditions and results obtained in a stirred reactor.

0069 Despite the aeration of the culture with saturated air, the medium dried out to such an extent that it was necessary to readjust the water content on two occasions, when it fell below 50%, as indicated in Table 5.

0070 The medium temperature could be maintained at an average value of 35°C by reducing the intake air and by intermittent stirring.

0071 Under these culture conditions, fungal growth - the development of which was monitored by measuring the pH - was maintained for 50 hours and production reached 1436 $GU.g^{-1}$ DM in 44 hours and 1990 $GU.g^{-1}$ DM in 63 hours. In relation to the initial dry mass, the quantities of GA produced were 1160 and 1540 $GU.g^{1-1}$ IDM respectively. For the purposes of comparison, we obtained 1805 $GU.g^{-1}$ DM during 44 hours in Example 3, which is equivalent to 1067 $GU.g^{-1}$ IDM. This information is interesting, as it indicates that the productivity of these two tests was identical, but that the experimental conditions in Example 4 permitted a longer enzyme production time, with a bed height amounting to 40 cm.

0072 Treating the bran with water vapour, followed by fermentation in the INRA 501 reactor, thus prolongs the fungal culture and the production of enzymes.

0073 In relation to the initial dry mass, the quantity of GA produced is $1540GU.g^{-1}$ IDM.

0074 Using the same samples, the dosaging of xylanolytic and proteolytic activities was carried out. The results obtained were very satisfactory, with the average maximum fermentation time of 50 hours resulting in:

- 350 $XU.g^{-1}$ IDM for xylanase;
- 400 $PU.g^{-1}$ IDM for protease.

0075 Because of the continuous recording of the mass, it was possible to calculate the loss of dry material during the culture. This was about 23% after 60 hours of culture time (within 2% weighing accuracy).

EXAMPLE 5 - The utilisation of fermented bran produced in Example 4 for the hydrolysis of wheat flours

0076 A series of saccharifications was carried out on wheat flour, using the fermented brans obtained in Example 4. The wheat flour had previously been subjected to traditional enzymatic liquefaction treatment. The preparation of AMG 300L$^{(R)}$ glucoamylase marketed by the company NOVO was used as a control. These tests were carried out with a conventional type 45 wheat flour. The operating conditions are summarised in Table 7 for 750 g of wort.

Table 7

Product	**AMG 300 L$^{(R)}$ (Novo)**	**Fermented bran**	**Dry fermented bran**
Reference	AMG 300 L	Example 4	Example 4
Presentation	Liquid	Humid bran	Dry bran
Storage method	at +5°C	at 20°C	at room temp.
Flour	Commercial type 45	Commercial type 45	Commercial type 45
Quantity used (g)	300	300	300
Dry material (%)	35	35	35
Liquefaction cond.	1h/88°C/pH 6,1	1h/88°C/pH 6,2	1h/88°C pH 6,2
Enzyme	125µl Termamyl. 120L$^{(R)}$	125µl Termamyl. 120 L$^{(R)}$	125µl Termamyl. 120 L$^{(R)}$
Saccharif. cond.	44h/58°C/pH 4,6	40h/58°C/pH 4,55	44h/60°C/pH 4,52
Qty equiv. to 3500 GU	205µl	4,3g	2,1g

0077 During these hydrolyses of the wheat flour, we took three samples of the medium each time. The results of the sugar concentrations at various moments of saccharification as shown in Table 8 are the average of these three samples. These dosages were determined according to the DNS technique and were carried out on the centrifuged samples. The final viscosity of the saccharified products was also measured.

Table 8

Measures	**AMG 300 L$^{(R)}$ (Novo)**	**Fermented bran**	**Dry fermented bran**
Initial sugar concentration (g/l)	180,9±4,6	185,0±3,1	171,5±4,5
Final sugar concentration (g/l)	327,5±18,5	325,0±22,5	348,3±19,1
Viscosity (mPa.s)	6,80	2,82	2,80

0078 In addition, an increase in the content of soluble nitrogen of the wort was found after saccharification, due to the proteolytic activity of the fermented bran.

0079 These results indicate that the fermented brans produced in Example 4 are

capable of hydrolysing the wheat flour with the same efficacy as a standard GA product, whichever mode of storage is used.

0080 The hydrolysis of the flour with its fermented bran also involves a significant reduction in viscosity, compared with a conventional enzyme product.

EXAMPLE 6

0081 This example illustrates the possibility of producing a considerable quantity of xylanase and a little glucoamylase using the *Aspergillus niger* stock.

0082 This test was carried out in the Fujiwara pilot fermentor, using BCE bran and *Aspergillus niger* stock ATCC 201202, which is known for its capacity to produce xylanase. The functions of the pilot fermentor are detailed in Example 3. Some 20 kg of medium with a humidity level of 55%, prepared as shown in Example 2, were used in this example. As in Example 3, during fermentation, the humidity of the medium was maintained at above 50% and the temperature of the medium was adjusted to about 35°C.

0083 Ultimately, the stock *Aspergillus niger* ATCC 201202 was produced after 37 hours under these fermentation conditions, with the fermented bran having 727 XU/g DM and 162 GU/g DM.

EXAMPLE 7: The advantages of incorporating fermented bran in accordance with the invention into poultry based on wheat destined to be chicken feed.

0084 It is known that the wheat flour hemicellulases are partially soluble in water and increase the viscosity of the intestinal contents, thus reducing the release and absorption of nutrients.

0085 The article by A. Veldman and H.A. Vahl: "Xylanase in broiler diets with differences in characteristics and content of wheat", Br Poult Sci 1994, September, 35(4), pages 537-550, describes experiments used to measure the effect of adding pure xylanase to wheat-based poultry food on the zootechnical performance of such birds.

0086 It was shown that the addition of hemicellulase results in their break-down, thus making it possible to reduce the viscosity of the intestinal content and improving the zootechnical performance of monogastric animals such as broilers, which were fed on food containing only wheat cereal.

0087 An experiment was carried out on 1 200 Ross broilers to show the effect of using fermented bran as a carrier of hemicellulase activity (xylanase), in comparison with an enzyme-free food and a food containing a standard source of xylanase, i.e. the product Avizyme. The foods were prepared in the quantities required to feed 4 batches of 300 chickens. Their composition is given in Table 9. The growth food (GR FO) was used during the first 21 days of rearing the chickens, after which it was replaced by finishing food (FI FO) for a period of 18 days.

Table 9

Foodstuff	GR FO	FI FO
Humidity (%)	10,8	11,4
Proteins (%)	21,3	19,1
Fats (%)	6,1	6,4

0088 Food 1 did not receive any enzymes. Foods 2 and 3 received 3 and 5 kg of fermented bran per tonne of food respectively. Food 4 received 0,5 kg Avizyme[(R)] per tonne of food.

0089 The results of this test after 39 days are summarised in Table 10.

Table 10

Foodstuff	1	2	3	4
Fermented bran according to the invention (kg/tonne)[a]	---	3,0	5,0	---
AVIZYME Finfeed (kg/tonne)[b]	---	---	---	0,6
Xylanase activity (XU/kg food)	---	1 700	2 840	1 620
Consumption index - 39 days [c]	1,775	1,748	1,738	1,745
Consumption reduction index (% food) [d]	---	1,52	2,08	1,69
Mortality (%)	2,3	2,0	3,0	2,3

[a] This fermented bran has a glucoamylolytic activity of 1000 GU/g of dry material, a proteolytic activity of 125 PU/g of dry material and a xylanolytic activity of 800 XU/g of dry material;
[b] Avizyme[(R)] is provided by the company Finfeed in Finland.
[c] ratio of weight of food consumed to weight gain;
[d] reduction in % by weight of food consumed in relation to the weight of the food (without enzyme) consumed

0090 The incorporation of fermented bran into poultry food (3 or 5kg/tonne) makes it possible significantly to reduce the consumption index. Under the test conditions, the utilisation of a dose of fermented bran of more than 3 kg/tonne appears to be of no practical significance. The improvements observed are comparable to those obtained with the commercial reference product Avizyme (0,6 kg/tonne). The utilisation of fermented bran does, however, have the advantage of being less expensive than the utilisation of the commercial enzyme product.

Table 5 - Physico-chemical parameters of FMS in a 50-litre fermentor with a stirrer for vapour-treated bran

Culture time (h)	Intake air temp. (°C)	Air flow (l/min)	Air, % relative humidity	Average environ-mental temp.	Average pH	Total mass	Treat-ment
0	35,0	314,0	94,6		5,70	18,30	
13	36,1	312,7	92,6	38,8	4,72	18,00	
13 (after stirring)		471,0		38,6	4,68		stirring
16	29,8	448,6	94,6	32,4	4,60	17,50	stirring
18	33,2	466,5	77,2	31,0	4,37	17,10	
20,33	32,6	466,5	93,7	34,3	4,20	16,80	
23,66				34,2	4,13	15,80	
26	29,8	467,5	94,6	32,9	4,17	15,10	
26	29,8	467,5	94,6	33,3	3,81	17,30	stirring + 2,5 l water
36,75	29,0	467,5	94,6				stirring
36,75	27,0	467,5	94,6	29,9	3,71	14,30	
40,58	27,0	467,5	94,6	31,0	4,20	13,10	
40,66	27,0	467,5	94,6	31,7	3,60	15,10	stirring + 2,1 l water
44,58		448,6		33,6	3,71	14,10	
47,17		467,5		34,5	4,04	13,30	
50	27,0	303,3	94,6	32,0	4,09	12,80	stirring
63,33	28,7	303,0		31,0	5,75	10,80	

Table 6: Summary of results of FMS in a 50-litre fermentor with a stirrer for vapour-treated bran

Cult. time (h)	Water content (%)	Loss of MS (%)	Loss of MS (%) (smooth)	UG/g MS - average	UG/g MSI - average	UX/g MS	UX/g MSI	UP/g MS	UP/g MSI
0	54,50	-2,07		0,00	0,00	0,0	0,0	0,0	0,0
13	57,52	7,78	0,4	92,69	85,48	16,1	16,0	131,0	130,49
16	53,72	2,11	1,0	170,53	166,92	19,2	19,0	196,2	194,15
18	55,23	7,21	1,8	129,25	222,01	27,9	27,4	103,9	102,02
20,33	51,90	1,91	3,1	429,36	421,18	35,0	33,9	193,7	187,63
23,66	50,88	5,37	5,9	551,93	521,21	51,0	48,0	283,4	266,81
26	49,06	6,15	8,2	651,45	611,36	75,4	69,2	296,5	272,14
26	57,76	10,57	8,2	548,42	490,44	54,1	49,7	262,1	240,57
36,75	49,27	10,75	18,4	1127,09	1005,88	203,5	166,2	359,1	298,20
40,58	51,14	20,55	20,5						
40,66	58,72	22,54	22,0	1278,58	990,35	227,3	180,7	347,9	276,52
44,58	54,10	19,21	22,6	1436,16	1160,27	408,3	318,6	367,1	286,45
47,17	54,01	23,07	23,2	1535,69	1181,48	511,5	395,7	496,8	384,30
50	54,86	26,67	24,4	1742,78	1277m91	429,8	330,1	628,2	482,48
63,33	44,69	22,62	22,6	1989,07	1539,07	410,3	310,1	478,3	361,5

Table 2

	Cult. time (h)	Intake air temp. (°C)	Air flow (l/min)	Air flow (m/s)	Air, % RH	Av. envir. temp.	Av. pH	% humi-dity	Av. UGA/g MS	Cult. time (h)
F4C3	0,0	35	17,4	8,0	96	25,0				0,0
dry bran	3,0	35	17,4	8,0	95	33,8	6,33			3,0
	11,0	32	17,4	8,0	94	34,6	5,13	54,53	20,56	11,0
	14,0	30	17,4	8,0	95	35,4	4,88	54,19	71,13	14,0
Scoring (17 h)	16,0	30	17,4	8,0	94	33,3	4,36	52,17	123,12	16,0
Scoring (18 h)	18,0	28	17,4	8,0	92	37,4	4,51	51,31	95,78	18,0
	20,0	28	17,4	8,0	96	31,8	3,79	49,54	132,81	20,0
	22,0	28	17,4	8,0	95	34,7	4,12	45,17	188,0	22,0
	25,0	28	17,4	8,0		37,6	4,34	45,13	181,03	25,0
	28,0	28	17,4	8,0	94	32,6	4,63	36,47	129,58	28,0
	31,0	28	17,4	8,0		32,8	5,05	30,68	196,10	31,0
	40,0	28	17,4	8,0	95	31,9	5,69	23,96	246,86	40,0

Table 3

	Cult. time (h)	Intake air temp. (°C)	Air flow (l/min)	Air flow (m/s)	Air, % RH	Av. envir. temp.	Av. pH	% humidity	Av. UGA/g MS	Cult. time (h)
F4C3	0,0	35	17,4	8,0	96	25,0	6,96			0,0
wet bran	3,0	35	17,4	8,0	95	33,8	6,12			3,0
	11,0	32	17,4	8,0	94	35,1	5,06	53,93	135,77	11,0
Scoring (14 h)	14,0	30	17,4	8,0	95	39,9	4,66	53,04	121,02	14,0
	16,0	30	17,4	8,0	94	33,1	4,59	51,53	182,96	16,0
Scoring (18 h)	18,0	28	17,4	8,0	92	38,0	4,48	50,80	327,41	18,0
	20,0	28	17,4	8,0	96	34,3	3,72	51,76	360,25	20,0
	22,0	28	17,4	8,0	95	36,9	3,70	47,65	618,00	22,0
	25,0	28	17,4	8,0		36,3	4,15	41,68	658,05	25,0
	28,0	28	17,4	8,0	94	30,9	4,53	35,78	660,03	28,0
	31,0	28	17,4	8,0		30,8	5,18	33,98	583,62	31,0
	40,0	28	17,4	8,0	95	30,2	4,88	30,02	702,74	40,0

Table 4

	Time (h)	Ventila-tion speed (rpm)	Air humi-dity (%)	Intake air temp. (°C)	Environ-mental air temp. (°C)	Humidity of environ-ment (%)	pH	UG/g MS	UG/g MSI	Time (h)
Stir.	0	15	99,0	35,0	25,0	54,0	6,36	4,3	4,3	0
	10	15	98,5	35,0	34,7	53,7	5,95	23,9		10
	12	15		33,0						12
	15	15	98,6	33,0	34,5	51,6	4,92	133,8		15
Stir. +3 l water	18	15	98,8	33,0	37,3	49,7	4,18	304,8	273,1	18
	21	15	98,3	30,0	30,7	54,7	3,83	472,1		21
	24	15	98,2	30,0	31,9	52,4	3,76	709,2		24
Stir. +2 l water	28	15	97,9	30,0	33,4	46,6	4,08	1198,0		28
	29	15	96,0	30,0		51,6		1054,0		29
Stir. +6 l water	38	15	98,6	32,0	31,5	39,2	5,00	1469,7	976,1	38
	39	15	99,0	32,0		60,6		1338,0		39
	42	15	99,2	32,0	33,9	59,7	5,28	1514,8		42
	44	15	98,1	32,0	34,1	54,8	5,76	1605,1	1067,6	44
	46	15	96,8	32,0	33,7	52,6	6,33	1601,0		46
	66	15	99,0	32,0	32,4	34,0	7,28	1594,4	1161,7	66

Claims

1. Composition exhibiting glucoamylase, proteolytic and xylanase activities, characterized in that it consists of wheat bran fermented with an *Aspergillus* strain chosen among the ATCC 201202, ATCC 76060, ATCC 76051, MUCL 28815, MUCL 28816, NRRL 3112 strains or with an *Aspergillus oryzae* strain chosen among the ATCC 22788, ATCC 42149 strains, said enzymatic glucoamylase, proteolytic and xylanase activities being present at the following minimum values:
   - glucoamylase: at least 100 GU per gramme of dry matter
   - proteolytic: at least 100 PU per gramme of dry matter
   - xylanase: at least 100 XU per gramme of dry matter,

   provided that the glucoamylase activity is at least 750 GU per gramme of dry matter and/or the xylanase activity is at least 300 XU per gramme of dry matter.

2. Composition according to claim 1, characterized in that it exhibits a glucoamylase activity of at least 750 GU/g of dry matter.

3. Composition according to claim 1, characterized in that it exhibits a glucoamylase activity of at least 1 500 GU/g of dry matter.

4. Composition according to claim 1, characterized in that it exhibits a xylanase activity of at least 300 XU per gramme of dry matter.

5. Product according to claim 1, characterized in that it exhibits a xylanase activity of at least 400 XU per gramme of dry matter.

6. Composition according to claim 1, characterized in that the *Aspergillus niger* strain is chosen from the NRRL 3112 strain, the ATCC 76061 strain.

7. Composition according to claim 6, characterized in that the Aspergillus niger strain is the ATCC 78091 strain.

8. Method for producing the composition defined in claim 1, the bran being in the form of a layer at least 10 cm thick, characterized in that it comprises the stages consisting in (a) taking wheat bran; (b) moistening and then heat-treating said bran so as to pasteurize it or sterilize it; (c) inoculating the resulting wheat bran with an *Aspergillus* strain chosen among the ATCC 201202, ATCC 76060, ATCC 76061, MUCL 28815, MUCL 28816, NRRL 3112 strains or with an *Aspergillus oryzae* strain chosen among the ATCC 22788, ATCC 42149 strains; (d) fermenting it in the solid state in a reactor which is aerated and stirred periodically for a period of 1 to 3 days, at a temperature of 28-38°C, said bran being adjusted to an initial moisture content of 50 to 60 wL%, which is substantially maintained during fermentation, under aeration conditions appropriate for avoiding accumulation of carbon dioxide, which is harmful to the fermentation in the reactor, and a rise in temperature due to fermentation above the recommended range, until the fermentation composition exhibits the following minimum enzyme activity values:
   - glucoamylase: at least 100 GU per gramme of dry matter

- proteolytic: at least 100 PU per gramme of dry matter
- xylanase: at least 100 XU per gramme of dry matter

provided that the glucoamylase activity is at least 750 GU per gramme of dry matter and/or the xylanase activity is at least 300 XU per gramme of dry matter.

9. Method according to claim 8, characterized in that the Aspergillus niger strain is chosen from the NRRL 3112 strain, the ATCC 76061 strain.

10. Method according to claim 9, characterized in that the Aspergillus niger strain is the ATCC 76061 strain.

11. Method according to any of claims 8 to 10, characterized in that an inoculation dose is at least 1.10^7 spores per gramme of initial dry matter.

12. Method according to any of claims 8 to 11, characterized in that it comprises the additional step consisting in freezing or drying the composition obtained in stage d).

13. Use of a product according to any of claims 1-3 and 4-7 in the production of ethanol from wheat.

14. Monogastric animal feed according to any of claims 1 and 4-7.